

## Original Article

# The vasorelaxing effect of hydrogen sulfide on isolated rat aortic rings versus pulmonary artery rings

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**Aim:** To compare the vasorelaxing effects of hydrogen sulfide (H<sub>2</sub>S) on isolated aortic and pulmonary artery rings and to determine their action mechanisms.

**Methods:** H<sub>2</sub>S-induced vasorelaxation of isolated rat aortic versus pulmonary artery rings under 95% O<sub>2</sub> and 5% CO<sub>2</sub> was analyzed. The expression of cystathionine gamma-lyase (CSE), cystathionine beta synthase (CBS), 3-mercaptopyruvate sulfurtransferase (3MST), SUR2B and Kir6.1 was examined.

**Results:** NaHS caused vasorelaxation of rat aortic and pulmonary artery rings in a dose-dependent manner. NaHS dilated aortic rings to a greater extent (16.4%, 38.4%, 64.1%, 84.3%, and 95.9% at concentrations of 50, 100, 200, 500, and 1000 μmol/L, respectively) than pulmonary artery rings (10.1%, 22.2%, 50.6%, 73.6%, and 84.6% at concentrations of 50, 100, 200, 500 and 1000 μmol/L, respectively). The EC<sub>50</sub> of the vasorelaxant effect for aortic rings was 152.17 μmol/L, whereas the EC<sub>50</sub> for pulmonary artery rings was 233.65 μmol/L. The vasorelaxing effect of H<sub>2</sub>S was markedly blocked by cellular and mitochondrial membrane K<sub>ATP</sub> channel blockers in aortic rings (*P*<0.01). In contrast, only the cellular membrane K<sub>ATP</sub> channel blocker inhibited H<sub>2</sub>S-induced vasorelaxation in pulmonary artery rings. SUR2B mRNA and protein expression was higher in aortic rings than in pulmonary artery rings. Cystathionine gamma-lyase (CSE) but not cystathionine beta synthase (CBS) expression in aortic rings was higher than in pulmonary artery rings. 3-Mercapto pyruvate sulfurtransferase (3MST) mRNA was lower in aortic rings than in pulmonary artery rings.

**Conclusion:** The vasorelaxing effect of H<sub>2</sub>S on isolated aortic rings was more pronounced than the effect on pulmonary artery rings at specific concentrations, which might be associated with increased expression of the K<sub>ATP</sub> channel subunit SUR2B.

**Keywords:** hydrogen sulfide; aortic rings; pulmonary rings; vasorelaxation; cystathionine gamma-lyase; cystathionine beta synthase; 3-mercapto pyruvate sulfurtransferase; glibenclamide, 5-hydroxydecanoate

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## Introduction

The regulation of systemic and pulmonary circulation is a very important issue in cardiovascular research. Systemic circulation differs from pulmonary circulation in several important aspects. The same pathological stimuli may elicit different responses from either systemic or pulmonary circulation. For example, under hypoxia (20–60 mmHg pO<sub>2</sub>), pulmonary arteries contract while systemic arteries relax<sup>[1]</sup>. Vasoactive substances such as endothelin (ET) and angiotensin II (Ang II) play very important roles in the cardiovascular system but induce different vascular responses in pulmonary and

systemic circulation<sup>[2–4]</sup>. The above-mentioned studies have revealed many differences in the responses of systemic and pulmonary circulation to pathophysiological stimuli.

In their carefully performed study, Olson *et al* examined vertebrate vessels and found that H<sub>2</sub>S produced temporally and quantitatively identical responses even though the responses varied from constriction (lamprey dorsal; IDA) to dilation (rat aorta; rA) to multiphasic (rat and bovine pulmonary arteries; rpA and bPA, respectively)<sup>[5]</sup>. They discovered that the concentration of vasoactive H<sub>2</sub>S in the vessel was governed by a balance between endogenous H<sub>2</sub>S production and its oxidation by available O<sub>2</sub><sup>[5]</sup>. In our study, we tried to analyze the difference between rat aorta and pulmonary artery at the vasorelaxant stage and further explored the role of K<sub>ATP</sub> channels in the regulation of the vasorelaxant effect by hydrogen sulfide.

The endogenous gaseous signaling molecule hydrogen

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sulfide ( $\text{H}_2\text{S}$ ) functions as a physiological regulator<sup>[6-12]</sup>. Recent studies have shown that cystathionine gamma-lyase (CSE), cystathionine beta synthase (CBS), and 3-mercapto-pyruvate sulfurtransferase (3MST) are three  $\text{H}_2\text{S}$  generating enzymes<sup>[13-14]</sup>. It is shown that  $\text{H}_2\text{S}$  relaxes blood vessels and lowers blood pressure by opening ATP-sensitive  $\text{K}^+$  channels in vascular smooth muscle cells<sup>[15]</sup>. To examine and compare the pathways used for the endogenous production of  $\text{H}_2\text{S}$  in aortic and pulmonary arteries, we tested the expression of the above-mentioned enzymes. It has been demonstrated that  $\text{H}_2\text{S}$  acts as an endogenous  $\text{K}_{\text{ATP}}$  channel opener to regulate vascular tone and that Kir6.1 and SUR2B are the main  $\text{K}_{\text{ATP}}$  channel subunits expressed in the vascular smooth muscle cells. So we tested the protein and mRNA expression of SUR2B and Kir6.1 in aortic and pulmonary arteries to investigate the possible mechanisms responsible for the regulation of vasorelaxation by  $\text{H}_2\text{S}$ .  $\text{H}_2\text{S}$  deficiency has been observed in animal models of systemic and pulmonary hypertension<sup>[16-18]</sup>. It also plays important roles in the pathogenesis of cardiovascular diseases<sup>[16-24]</sup>. The main mechanism for the cardiovascular actions of  $\text{H}_2\text{S}$  was considered to be the activation of  $\text{K}_{\text{ATP}}$  channels, because  $\text{H}_2\text{S}$  increased whole-cell  $\text{K}_{\text{ATP}}$  channel currents in rat aortic vascular smooth muscle cells<sup>[15]</sup>. However, whether  $\text{H}_2\text{S}$  exerts different vasorelaxing effects on aortic and pulmonary artery rings is unknown. If it does, the potential mechanisms behind these effects are not understood. Therefore, this study was designed to observe the vasorelaxing effect of  $\text{H}_2\text{S}$  on isolated aortic and pulmonary artery rings of rats *in vitro* and to identify the possible mechanisms.

## Materials and methods

### Reagents

Glibenclamide (Gli), 5-hydroxydecanoate (5-HD), and nica-dipine were purchased from American Sigma Aldrich Company. NaHS was dissolved in deionized water and freshly prepared solution was used.

### Animal preparation

The animal experimental procedures conformed to the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH) in the United States and was approved by the Animal Research Committee of Peking University. Adult male Wistar-Kyoto (WKY) rats weighing 220-250 g were purchased from Vital River (Beijing, China). Rats were housed in cages and fed a standard laboratory diet and fresh water. The cages were kept in a room with controlled temperature ( $24 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ ), relative humidity (65%-70%), and a 12-h light/dark cycle.

### Preparation for aortic and pulmonary artery rings

Male Wistar rats ( $n=10$ ) were anesthetized with urethane (1 g/kg body weight) intraperitoneally. The thoracic cavity was opened quickly, and the thoracic aorta and pulmonary artery were rapidly dissected and cleaned from fat and connective tissues. The artery was separated as carefully as possible to maintain the vascular activity. Rings 2-3 mm in length

were cut and placed in  $0 \text{ }^\circ\text{C}$  Krebs solution and immersed in 20 mL of organ baths containing pre-warmed Krebs' bicarbonate buffer filled with 95%  $\text{O}_2$ -5%  $\text{CO}_2$  at  $37 \text{ }^\circ\text{C}$ . The composition of the Krebs solution was as follows (mmol/L): NaCl: 120, KCl: 5.5,  $\text{CaCl}_2$ : 2.5,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ : 1.2,  $\text{NaH}_2\text{PO}_4$ : 1.2,  $\text{NaHCO}_3$ : 20, EDTA- $\text{Na}_2$ : 0.03, glucose: 10, and pH: 7.2-7.4. Organ baths were filled with oxygenated (95%  $\text{O}_2$ -5%  $\text{CO}_2$ ) Krebs solution.

Changes in tension were recorded using force transducers connected to a PowerLab (BL Newcentrany, TaiMeng, Chengdu, China). First, the aortic rings were stretched passively to a tension of 1 g, while the pulmonary rings were stretched at 0.6 g. The rings were equilibrated for 1 h before starting the experiment. The endothelia of the rings were kept functionally unbroken, as confirmed by their relaxation after acetylcholine treatment (1  $\mu\text{mol/L}$ ). The rings were contracted with norepinephrine (NE, 1  $\mu\text{mol/L}$ ). When the vasoconstriction curves of the rings reached the plateau phase of maximum tension,  $\text{H}_2\text{S}$  at 50-1000  $\mu\text{mol/L}$  was given and the changes in tension were recorded. In another experiment, aortic and pulmonary artery rings were incubated with two kinds of  $\text{K}_{\text{ATP}}$  channel blockers for 30 min (at a concentration of  $1 \times 10^{-6}$  mol/L) before the physiological dose of 100  $\mu\text{mol/L}$  NaHS was given to observe whether the vasorelaxing effect of  $\text{H}_2\text{S}$  could be blocked. The relaxation ratio was calculated by the relaxation degree and preshrinking degree and expressed as a percentage (%). The relaxation degree and shrinking degree, in grams, were recorded by electrophysiological graph.

### Measurement of CSE, CBS, SUR2B and Kir6.1 expression in aortic and pulmonary artery rings by Western blotting

Aortic and pulmonary artery rings from Wistar rats ( $n=10$ ) were homogenized and lysed. Equal amounts of proteins were boiled and separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes according to the experimental protocol. The primary antibody dilutions were 1:1000 for CSE, 1:4000 for CBS, 1:500 for SUR2B, 1:200 for Kir6.1, and 1:4000 for GAPDH antibodies. Secondary antibody (Santa Cruz) was used at a 1:10000 dilution. The immunoreactions were visualized by electrochemiluminescence (ECL) and exposed to X-ray film (Kodak Scientific Imaging film).

### Measurement of CSE, SUR2B and Kir6.1 expression in aortic and pulmonary rings by immunohistochemistry

After dewaxing by dimethylbenzene, sections of aortic and pulmonary artery rings were placed in distilled water and treated with 3%  $\text{H}_2\text{O}_2$  for 12 min. The slides were washed with PBS three times for 5 min each. The antigens were then exposed for 15 min. The slides were rinsed again, and the samples were blocked for 30 min with goat serum working fluid. Polyclonal antibodies to CSE (1:150), SUR2B (1:50), and Kir6.1 (1:50) were added and incubated at  $4 \text{ }^\circ\text{C}$  overnight. On the following day, slides were rinsed three times for 5 min in PBS and then incubated with biotinylated anti-mouse, goat, or rabbit IgG at  $37 \text{ }^\circ\text{C}$  for 60 min. Slides were rinsed again in PBS three times, and horseradish peroxidase streptavidin was added for 30 min at  $37 \text{ }^\circ\text{C}$ , followed by three 5 min-washes

with PBS. DAB was added for color development, and the sections were counter-stained with hematoxylin. The sections were dehydrated through a graded ethanol series, treated with dimethylbenzene, and then mounted on slides. The presence of brown granules in aortic and pulmonary smooth muscle cells and endothelial cells was defined as positive signal. For negative controls, sections were processed as described above except that the primary incubation was performed with non-immune goat serum instead of primary antibodies.

#### Measurement of SUR2B, Kir6.1, and 3MST mRNA expression in aortic and pulmonary artery rings using quantitative real-time polymerase chain reaction (PCR)

RNA from aortic and pulmonary artery rings of rats ( $n=7$ ) was extracted using Trizol reagent (GibcoBRL) and reverse transcribed using an oligo d(T)18 primer and M-MLV reverse transcriptase. Primers and TaqMan probes used for the quantification of cDNAs in samples were designed using the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). Primers and probes were synthesized by the SBS Company, Limited (Beijing, China). Quantitative real-time PCR was carried out using an ABI PRISM 7300 instrument (Applied Biosystems). The sequences of the primers and probes are shown in Table 1. The PCR condition for SUR2B, Kir6.1 and 3MST were as follows: pre-denaturation at 94 °C for 5 min, then 94 °C for 30 s, 59.5 °C for 30 s, and 70 °C for 1 min for 40 cycles. The PCR condition for  $\beta$ -actin was 95 °C for 5 min, 95 °C for 15 s, and 60 °C for 1 min for 40 cycles. The amount of  $\beta$ -actin cDNA in the sample was used to calibrate the amount of sample needed for quantification.

#### Statistical analysis

The data were analyzed by Excel and SPSS 13.0 statistical software, and all values were expressed as mean $\pm$ standard deviation. The relaxation reaction at different concentrations of NaHS on aortic and pulmonary artery rings was analyzed by an independent sample *t*-test. The relaxation reaction to treatment of aortic and pulmonary artery rings with physi-

ological concentrations of NaHS (the WKY+NaHS, Gli+NaHS, and 5-HD+NaHS groups) was analyzed by one-way ANOVA. LSD analysis was used for comparing data between the two groups. The expression of SUR2B, Kir6.1, CSE, CBS, and 3MST in aortic and pulmonary arteries was compared using the paired-sample *t* analysis. A level of  $P<0.05$  was set as statistically significant.

## Results

#### The maximum diastolic effect of aortic and pulmonary artery rings to different concentrations of NaHS in rats

NaHS caused vasorelaxation in rat thoracic aortic and pulmonary artery rings pre-contracted with 1  $\mu\text{mol/L}$  NE *in vitro* in a dose-dependent manner.  $\text{H}_2\text{S}$  at concentrations of 50–1000  $\mu\text{mol/L}$  dilated aortic rings more noticeably than pulmonary artery rings ( $P<0.05$ , Figure 1). The  $\text{EC}_{50}$  of the vasorelaxant effect on aortic rings was 152.17  $\mu\text{mol/L}$ , while the effect on pulmonary artery rings had an  $\text{EC}_{50}$  of 233.65  $\mu\text{mol/L}$ .

#### The effects of a cytomembrane $\text{K}_{\text{ATP}}$ channel blocker and mitochondrial membrane $\text{K}_{\text{ATP}}$ channel blocker on the vasorelaxing effect of $\text{H}_2\text{S}$ on aortic and pulmonary artery rings

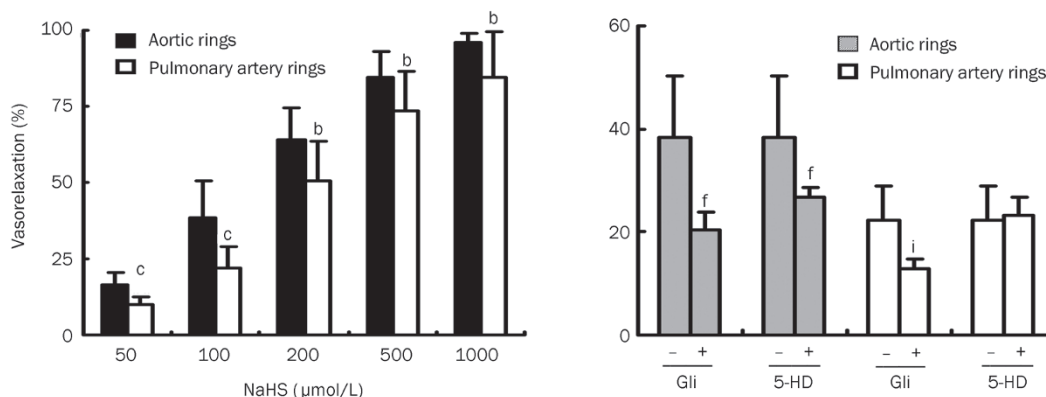
The vasorelaxing effect of  $\text{H}_2\text{S}$  was markedly blocked by cytomembrane and mitochondrial membrane  $\text{K}_{\text{ATP}}$  channel blockers (Gli and 5-HD) in aortic rings ( $P<0.01$ , Figure 1). In contrast, the  $\text{H}_2\text{S}$ -induced vasorelaxing effect on pulmonary artery rings could only be blocked by the cytomembrane  $\text{K}_{\text{ATP}}$  channel blocker ( $P<0.01$ , Figure 1) but not by the mitochondrial membrane  $\text{K}_{\text{ATP}}$  channel blocker ( $P>0.05$ , Figure 1).

#### The different vasoactive response of aortic and pulmonary artery rings to different concentrations of NaHS in rats

Within 30 min, all concentrations of hydrogen sulfide (50–1000  $\mu\text{mol/L}$ ) gradually relaxed the rat aorta over time until it reached its maximum level of vasorelaxation. However, in rat pulmonary arteries, NaHS (concentrations of 50, 100 and 200  $\mu\text{mol/L}$ ) produced a constriction followed by a relaxation,

**Table 1.** The sequence of the primers and probes of SUR2B, Kir6.1, 3MST and  $\beta$ -actin.

SUR2B	Forward primer	SUR2B-F: 5'-ACCCGCGAGTACAACCTTCTT-3'
	Reverse primer	SUR2B-R: 5'-TTCATCGCTCAAGAGAACTCAT-3'
	Probe	SUR2B-P: 5'-AGCCATCATCAGCGTTTCCAGAAGCT-3'
Kir6.1	Forward primer	Kir6.1-F: 5'-ACCCGCGAGTACAACCTTCTT-3'
	Reverse primer	Kir6.1-R: 5'-TATCGTCATCCATGGCGAACT-3'
	Probe	Kir6.1-P: 5'-AGGTCATTCACCTTCTGCGTTTCTCTTCCAT-3'
3MST	Forward primer	3MST-F: 5'-CGGCGCTTCCAGGTAGTG-3'
	Reverse primer	3MST-R: 5'-CTGGTCAGGAATTCAGTGAATGG-3'
	Probe	3MST-P: 5'-CGCAGCTGGCCGTTTCCA-3'
$\beta$ -Actin	Forward primer	$\beta$ -Actin-F: 5'-ACCCGCGAGTACAACCTTCTT-3'
	Reverse primer	$\beta$ -Actin-R: 5'-TATCGTCATCCATGGCGAACT-3'
	Probe	$\beta$ -Actin-P: 5'-CCTCCGTCGCGGTCCACAC-3'



**Figure 1.** The maximum relaxation response of aortic and pulmonary artery rings to different concentrations of NaHS in rats, and the effect of a  $K_{ATP}$  channel blocker on the vasorelaxing effect of hydrogen sulfide on aortic and pulmonary artery rings ( $n=10$ ). <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  compared to aortic rings. <sup>f</sup> $P<0.01$  compared to aortic rings without giving  $K_{ATP}$  channel blocker. <sup>i</sup> $P<0.01$  compared to pulmonary artery rings without giving  $K_{ATP}$  channel blocker. Gli: glibenclamide, 5-HD: 5-hydroxydecanoate.

and then followed by a reduced relaxation. This effect was not obvious at higher concentrations (500 and 1000  $\mu\text{mol/L}$ ) in rat pulmonary arteries (Figure 2).

#### Immunohistochemical analysis of CSE expression in aortic and pulmonary artery rings

CSE protein in aortic and pulmonary artery rings of rats in the control group was strongly expressed in the inner membrane and tunica media vasorum. The presence of brown granules in aortic and pulmonary artery smooth muscle cells and endothelial cells was defined as a positive signal (Figure 3).

#### CSE and CBS expressions in aortic and pulmonary artery rings by Western blotting

Compared to that in pulmonary artery rings, the expression of CSE protein in aortic rings was notably enhanced ( $P<0.05$ , Figure 4). However, there was no difference in CBS protein expression between aortic and pulmonary artery rings ( $P>0.05$ , Figure 4).

#### Expression of SUR2B, Kir6.1 and 3MST by real-time PCR

As measured by semi-quantitative real-time PCR, SUR2B mRNA was higher in aortic rings than in pulmonary artery rings ( $P<0.05$ , Figure 5). However, Kir6.1 mRNA expression did not differ between aortic rings and pulmonary artery rings ( $P>0.05$ , Figure 5). 3MST mRNA was lower in aortic rings than in pulmonary artery rings ( $P<0.05$ , Figure 5).

#### Immunohistochemical analysis of SUR2B and Kir6.1 expression in aortic and pulmonary artery rings

The SUR2B and Kir6.1 proteins in aortic and pulmonary artery rings from WKY rats were mainly expressed in the medial layer of the vessel. The brown granules in both aortic and pulmonary artery smooth muscle cells and endothelial cells viewed were defined as positive signals (Figure 6).

#### SUR2B and Kir6.1 expression in aortic and pulmonary artery rings as shown by Western blotting

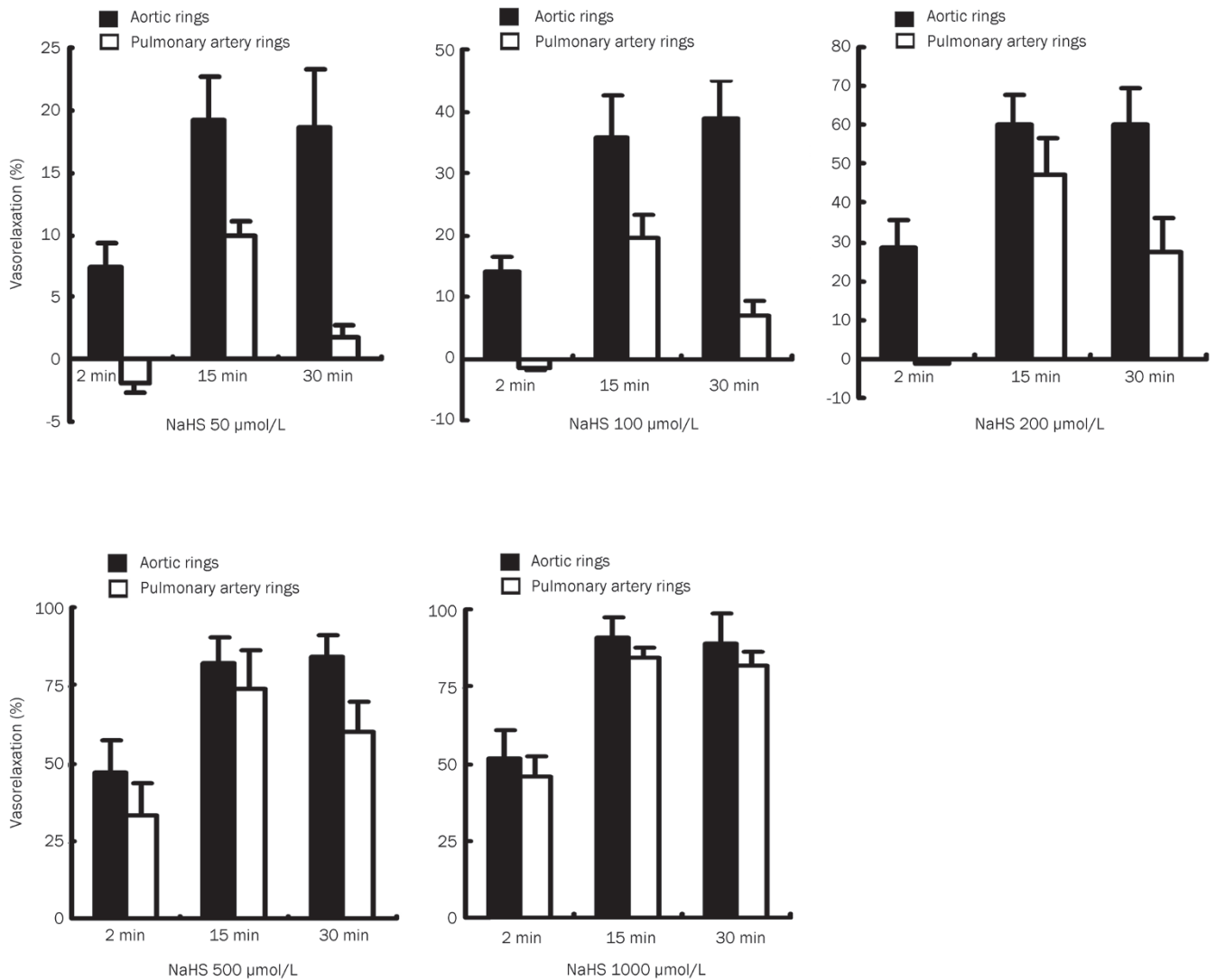
Compared to the pulmonary artery rings, the expression of SUR2B protein increased in the aortic rings of the Wistar rats ( $P<0.05$ , Figure 4), but there was no difference in Kir6.1 protein expression between aortic and pulmonary artery rings ( $P>0.05$ , Figure 4).

#### Discussion

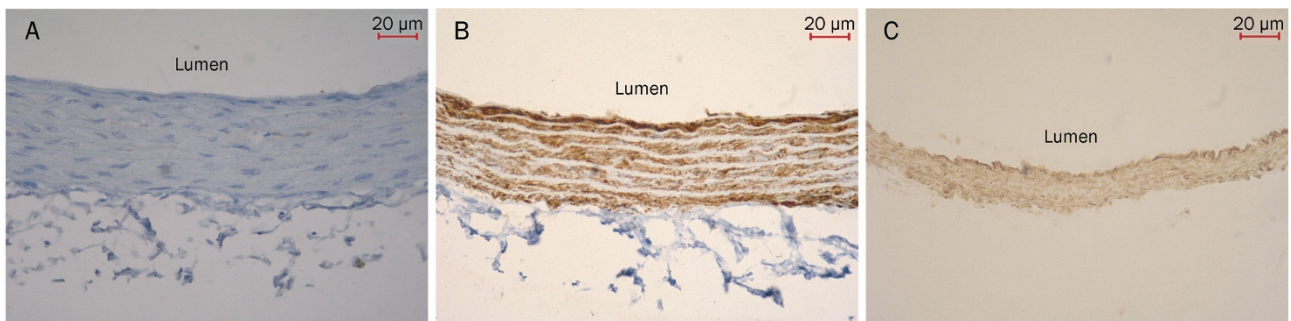
In the present study we found that NaHS resulted in the vasorelaxation of rat thoracic aortic rings in a dose-dependent manner, which was more pronounced than the vasorelaxation that occurred in pulmonary artery rings. In aortic rings, both cellular and mitochondrial membrane  $K_{ATP}$  channel blockers markedly inhibited  $\text{H}_2\text{S}$ -induced vasorelaxation. In contrast,  $\text{H}_2\text{S}$ -induced vasorelaxation in pulmonary artery rings could only be blocked by a cellular, but not mitochondrial, membrane  $K_{ATP}$  channel blocker. The expression of SUR2B protein and mRNA in aortic rings increased compared to pulmonary artery rings.

$\text{H}_2\text{S}$ , a novel gaseous signaling molecule, has been considered to play an important role in the regulation of cardiovascular functions<sup>[6-10]</sup>. Endogenous cardiovascular  $\text{H}_2\text{S}$  is mainly produced by CSE<sup>[8-10]</sup>. Recent evidence from CSE knockout mice suggests that loss of CSE gene expression results in a decrease in  $\text{H}_2\text{S}$  production and a subsequent rise in blood pressure<sup>[16]</sup>. Furthermore, Shibuya *et al* and Olson *et al* showed that CSE, CBS, and 3MST were the three important  $\text{H}_2\text{S}$  generating enzymes<sup>[13, 14]</sup>. In our study, we found that the mRNA expression of 3MST in rat aorta was lower than that in the pulmonary artery, whereas the CSE protein was higher in the rat aorta than in the pulmonary artery. However, there was no difference in CBS protein expression in the rat aorta and pulmonary artery.

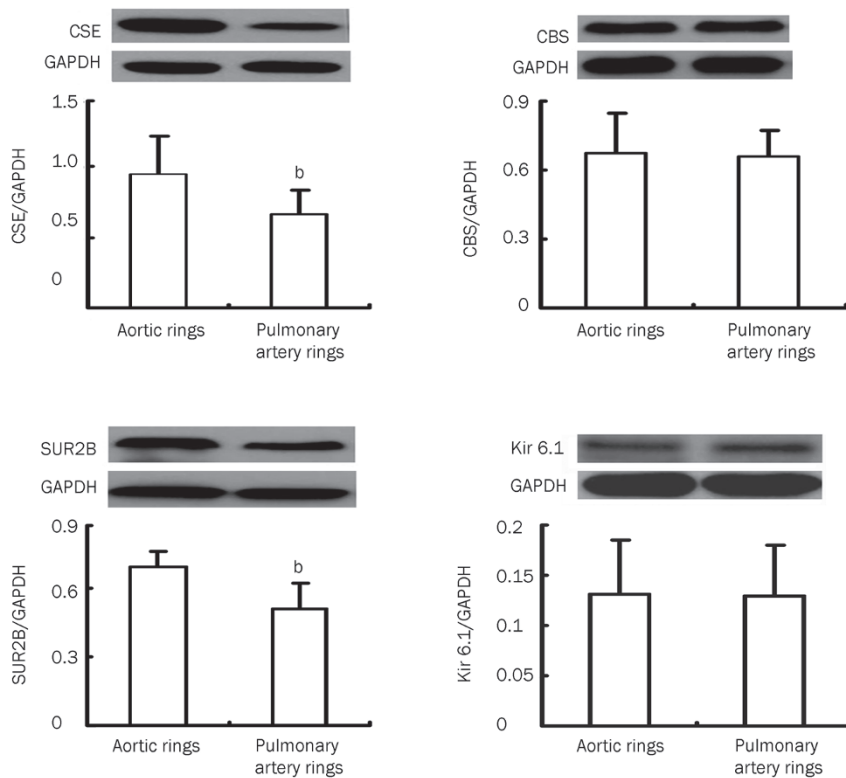
$\text{H}_2\text{S}$  plays an important role in the regulation of systemic



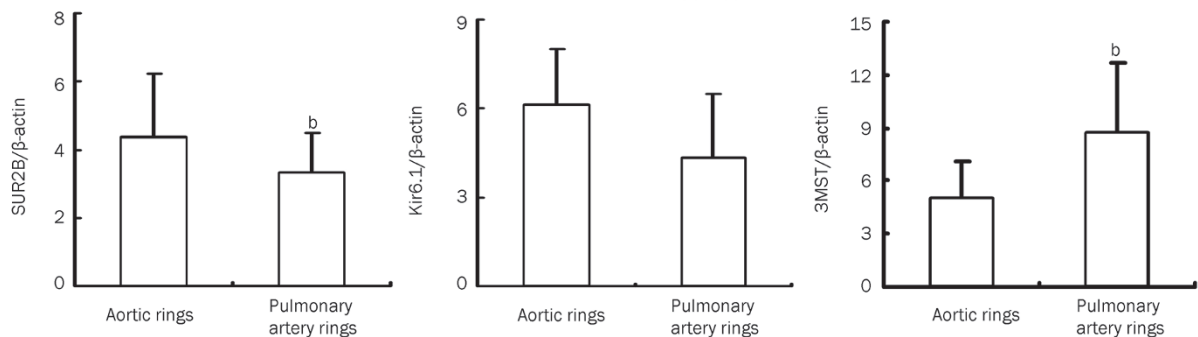
**Figure 2.** The different vasoactive response of aortic and pulmonary artery rings to different concentrations of NaHS in rats at the different time points.



**Figure 3.** Immunohistochemical analysis of CSE expression in aortic and pulmonary artery rings (DAB×200). (A) The aortic ring negative control was processed without CSE primary antibody. The thickness of the inner elastic layer was uniform and the structure of smooth muscle cells was normal. (B) The aortic ring was processed with CSE antibody. CSE protein was strongly expressed in the inner membrane and tunica media vasorum. The brown granules in aortic smooth muscle cells and endothelial cells were defined as positive signals. (C) The pulmonary artery ring was processed with CSE antibody. The brown granules were observed in pulmonary artery smooth muscle cells and endothelial cells. CSE, cystathionine gamma-lyase.



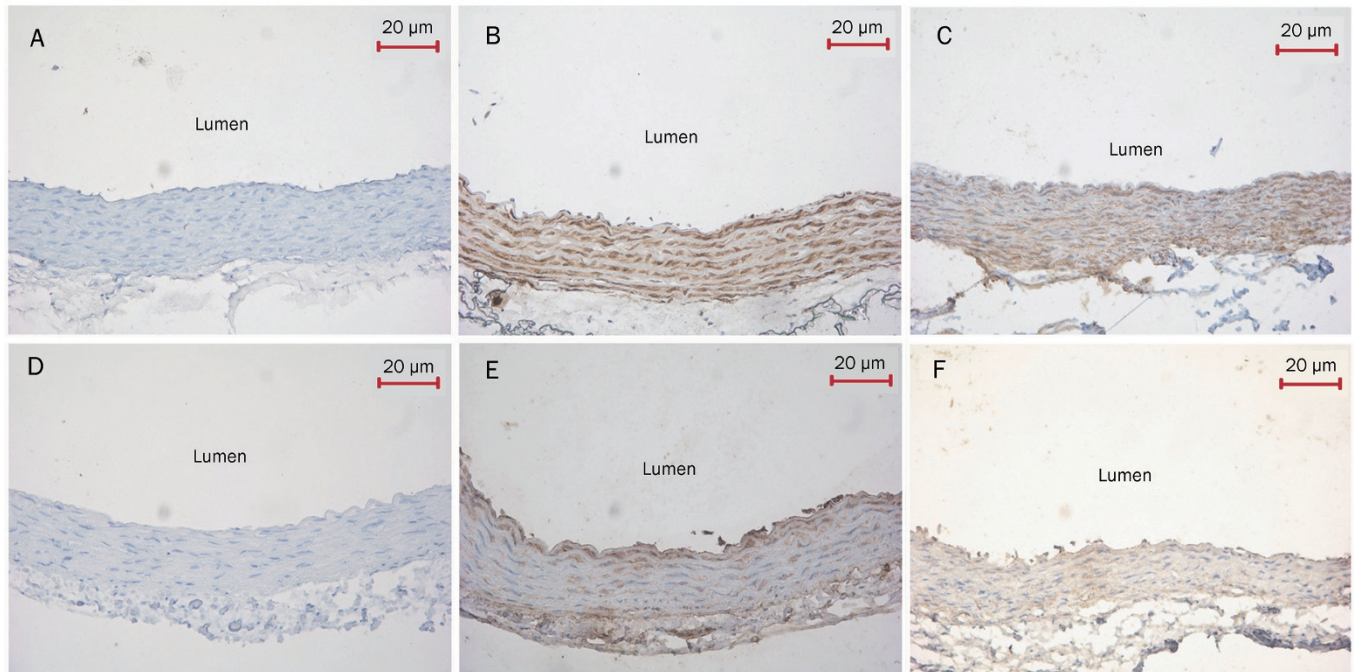
**Figure 4.** CSE, CBS, SUR2B and Kir6.1 expression in aortic and pulmonary artery rings as detected by Western blotting ( $n=10$ , mean $\pm$ SD). <sup>b</sup> $P<0.05$  compared to aortic rings. CSE, cystathionine gamma-lyase; CBS, cystathionine beta synthase; SUR2B, a  $K_{ATP}$  channel subunit; Kir6.1, a  $K_{ATP}$  channel subunit.



**Figure 5.** Expression of SUR2B, Kir6.1 and 3MST by real-time PCR ( $n=7$ , mean $\pm$ SD). <sup>b</sup> $P<0.05$  compared to aortic rings. SUR2B, a  $K_{ATP}$  channel subunit. Kir6.1, a  $K_{ATP}$  channel subunit. 3MST, 3-mercaptopyruvate sulfurtransferase.

and pulmonary circulation<sup>[25-30]</sup>. Olson *et al* showed that in rat pulmonary arteries, NaHS produced a transit constriction followed by relaxation for 20 to 30 min, which was then followed by a second constriction<sup>[5]</sup>. In a later study, this same group carefully examined the effects of Na<sub>2</sub>S on the conductance and resistance responses of the cow and sea lion pulmonary arteries and showed that the sea lion arteries had vasodilating characteristics<sup>[31]</sup>. We analyzed the vascular response to H<sub>2</sub>S for 30 min. We found that in rat pulmonary arteries, NaHS at concentrations of 50, 100, and 200  $\mu$ mol/L produced a transit constriction followed by a relaxation for about 20 min, which was then followed by a reduced relaxation. However, this did not occur in the rat aorta.

In this study, we found that NaHS caused vasorelaxation of rat thoracic aortic and pulmonary artery rings pre-contracted with 1  $\mu$ mol/L NE *in vitro* in a dose-dependent manner. The mechanism for the vasoconstrictive response to norepinephrine is the action of NE on the vascular alpha adrenaline receptors, resulting in the vasoconstrictive response. A previous study showed that in sheep *in vivo*, the vasoconstrictor response to alpha-adrenergic stimulation was less in the pulmonary circulation compared to the systemic circulation of the fetus<sup>[32]</sup>. This same study also indicated that alpha-adrenergic receptor density was less pronounced in fetal intrapulmonary vascular smooth muscle than that in fetal aortic VSM<sup>[32]</sup>. The vasorelaxing effect of H<sub>2</sub>S on aortic and pulmonary rings is dependent



**Figure 6.** Immunohistochemical analysis of SUR2B and Kir6.1 expression in aortic and pulmonary artery rings (DAB×200). (A) The aortic ring negative control was processed without SUR2B primary antibody. This control had normal smooth muscle cell and endothelial cell structure without brown granules. (B) The aortic ring was treated with SUR2B antibody. SUR2B protein was strongly expressed in the inner membrane and tunica media vasorum. The presence of the brown granules in aortic smooth muscle cells and endothelial cells was defined as positive signals. (C) The pulmonary artery ring was processed with SUR2B antibody. Brown granules were observed in pulmonary artery smooth muscle cells and endothelial cells. (D) The aortic ring negative control was treated without Kir6.1 primary antibody. The structure of smooth muscle cells and endothelial cells was normal and had no brown granules. (E) The aortic ring was treated with Kir6.1 antibody. Brown granules were strongly expressed in the medial layer of aortic ring. (F) The pulmonary artery ring was processed with Kir6.1 antibody. The brown granules were observed in pulmonary artery smooth muscle cells and endothelial cells. SUR2B, a  $K_{ATP}$  channel subunit. Kir6.1, a  $K_{ATP}$  channel subunit.

on this initial pre-contraction. As far as we know, the differences in the vasorelaxing effects of  $H_2S$  between the aortic and pulmonary rings involve the following mechanisms: the mechanical properties of the blood vessels, the targeting ion channel  $K_{ATP}$  expressions and density where  $H_2S$  acts on. Thus, in our study, we attempted to examine if there were any  $K_{ATP}$  expression-mediated mechanisms in which  $H_2S$  acts on the different arteries.  $H_2S$  at concentrations of 50–1000  $\mu\text{mol/L}$  dilated aortic rings more significantly than pulmonary artery rings. This result indicates that  $H_2S$  at the same dose induces a stronger vasorelaxing effect in aortic rings compared to pulmonary artery rings.

$H_2S$  acts as a regulator of cardiovascular function<sup>[33, 34]</sup>. The opening of smooth  $K_{ATP}$  channels by  $H_2S$  has been suggested to be one of the mechanisms responsible for  $H_2S$ -induced vasorelaxation in vascular smooth muscle both *in vitro* and *in vivo*<sup>[24]</sup>.  $H_2S$  can open  $K_{ATP}$  channels in the cell membrane of aortic vascular smooth muscle, causing cytomembrane hyperpolarization. The  $K_{ATP}$  channel is very important in the cardiovascular system<sup>[35–41]</sup> and  $H_2S$  acts as an endogenous  $K_{ATP}$  channel opener.  $K_{ATP}$  channels are recognized for their cardioprotective role in ischemia<sup>[35]</sup>. Evidence suggests that Kir6.1 and SUR2B are the main  $K_{ATP}$  channel subunits expressed in the vascular smooth muscle<sup>[42–44]</sup>.

Therefore, we investigated the possible mechanisms responsible for the differences in vasorelaxation between aortic and pulmonary artery rings induced by  $H_2S$  by targeting  $K_{ATP}$  channels using cell and mitochondrial membrane  $K_{ATP}$  channel blockers. The results showed that cellular (Gli) and mitochondrial (5-HD) membrane  $K_{ATP}$  channel blockers could block  $H_2S$ -induced vasorelaxation in aortic rings. In contrast, in pulmonary artery rings, only the cell membrane  $K_{ATP}$  channel blocker effectively blocked  $H_2S$ -induced vasorelaxation. In aortic rings, vasorelaxation by NaHS was 38.4% at a concentration of 100  $\mu\text{mol/L}$ , which was reduced to 20.4% and 26.9% when aortic rings were pre-treated with cell and mitochondrial membrane  $K_{ATP}$  channel blockers, respectively. In pulmonary artery rings, the percent of vasorelaxation was 22.2% following 100  $\mu\text{mol/L}$  NaHS, which was reduced to 12.8% when pre-treated with the cell membrane  $K_{ATP}$  channel blocker. However, pre-treatment with the mitochondrial membrane  $K_{ATP}$  channel blocker did not alter pulmonary artery ring vasorelaxation. We presume that  $H_2S$  likely induces more obvious vasorelaxation in aortic rings because  $H_2S$  opens the  $K_{ATP}$  channels more widely in aortic rings than in pulmonary artery rings.

Next, we further examined whether there was any differences in  $K_{ATP}$  channel expression between aortic and pulmo-

nary artery rings. The results showed that protein expression of the  $K_{ATP}$  channel subunit SUR2B was higher in aortic than pulmonary artery rings. Furthermore, the mRNA expression of SUR2B was higher in aortic rings than in pulmonary artery rings. These findings suggested that the relatively higher density of  $K_{ATP}$  channels in aortic rings was partly responsible for the pronounced vasorelaxation observed in isolated aortic rings compared to those observed in pulmonary artery rings at specific concentrations. The identification of more profound mechanisms involved in the  $H_2S$ -induced vasorelaxation of aortic and pulmonary artery rings requires further investigation.

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### Author contribution

Jun-bao DU and Hong-fang JIN designed the research; Yan SUN and Hong-fang JIN performed the research and contributed new analytical reagents and tools; Yan SUN, Hong-fang JIN, Chao-shu TANG, and Jun-bao DU analyzed data; and Yan SUN, Hong-fang JIN, and Jun-bao DU wrote the paper.

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